

Regulation of neurite outgrowth by $G_{i/o}$ signaling pathways

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1. ABSTRACT

Neurogenesis is a long and winding journey. A neural progenitor cell migrates long distances, differentiates by forming a single axon and multiple dendrites, undergoes maturation, and ultimately survives. The initial formation of neurites during neuronal differentiation, commonly referred to as “neurite outgrowth,” can be induced by a large repertoire of signals that stimulate an array of receptors and downstream signaling pathways. The $G_{i/o}$ family of heterotrimeric G-proteins are abundantly expressed in the brain and enriched at neuronal growth cones. Recent evidence has uncovered several $G_{i/o}$ -coupled receptors that induce neurite outgrowth and has begun to elucidate the underlying molecular mechanisms. Emerging data suggests that signals from several $G_{i/o}$ -coupled receptors converge at the transcription factor STAT3 to regulate neurite outgrowth and at Rac1 and Cdc42 to regulate cytoskeletal reorganization. Physiologically, signaling through $G_{i/o}$ -coupled cannabinoid receptors is critical for proper central nervous system development. As the mechanisms by which $G_{i/o}$ -coupled receptors regulate neurite outgrowth are clarified, it is becoming evident that modulating signals from $G_{i/o}$ and their receptors has great potential for the treatment of neurodegenerative diseases.

2. INTRODUCTION

Neuronal differentiation is a complex process that integrates many signals to drive electrophysiological, morphological, and transcriptional changes (1,2,3,4). This process is characterized by the initial formation of immature neurites, commonly referred to as “neurite outgrowth.” The neurites then further develop into a single axon and multiple dendrites, followed by maturation of the neuron and the formation of dendritic spines (1). Many signals at the cell surface are integrated to shape axonal and dendritic outgrowths as well as their directionality and maturation (5,6,7,8). Not surprisingly, neurite outgrowth is precisely regulated due to its importance in the proper development of the organism. During neurite outgrowth, signals at the membrane are transduced to a large repertoire of enzymes to ultimately trigger changes in gene transcription in the nucleus. In addition, the signals produce vast changes in the actin and microtubule cytoskeletal networks in order to generate and stabilize the growing neurites (1,8).

A diverse array of ligands including neurotrophins, cytokines, hormones, and neurotransmitters can stimulate neurite outgrowth upon binding to their cognate receptors (5,7,9,10,11,12,13,14). Heterotrimeric

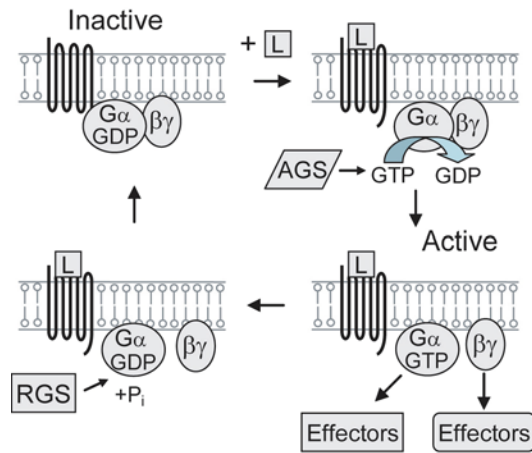


Figure 1. Heterotrimeric G-protein mechanism. The activation of the G-protein coupled receptor by a ligand (L) causes the exchange of GDP for GTP on the α subunit. This switches $G\alpha$ to the active conformation and results in the release of $G\alpha$ and $G\beta\gamma$ from the receptor to signal to downstream effectors. The switch is turned off by the intrinsic GTP hydrolysis (GTPase) activity of $G\alpha$, which leads to its reassociation with $G\beta\gamma$ and the receptor. The regulators of G-protein signaling (RGS) play key roles in inactivating G-protein signaling. The activators of G-protein signaling (AGS) activate G-proteins by several mechanisms.

guanine nucleotide-binding proteins (G-proteins) are one of the most widely used signal transduction systems in mammals, and signaling through the $G_{i/o}$ family of G-proteins regulates neurite outgrowth. In cortical neurons, dopamine-mediated stimulation of the D2 dopamine receptor can induce axonal neurite elongation (10). Similarly, activation of the serotonin (5-HT) 1B receptor by serotonin can enhance neurite outgrowth in thalamic neurons (11). While the activation of the adenosine 1A receptor in striatal neuronal precursor cells can induce neurite outgrowth (15), the activation of this receptor in hippocampal neurons appears to have an inhibitory role (16). G-proteins and their coupled receptors relay signals from the plasma membrane to downstream effectors in order to shape cell signaling pathways (17,18,19). By doing so, they regulate a vast array of cellular processes including metabolic enzyme activity, ion channel function, motility, transcription, and differentiation (17,18,19,20). This review will focus on signals from $G_{i/o}$ -coupled receptors that regulate neurite outgrowth during neuronal differentiation and their physiological and pathological implications.

3. $G_{i/o}$ SIGNALING

The molecular signals of many hormones, neurotransmitters, and chemokines are converted into intracellular responses by G-protein coupled receptors (17,18,19). These receptors generally consist of a seven transmembrane protein that is associated with a heterotrimeric G-protein which, as its name implies, is composed of three subunits: an α subunit ($G\alpha$) containing a Ras-like domain that can strongly bind the guanine nucleotides GTP and GDP, and beta and gamma subunits ($G\beta\gamma$) that function as a dimer and cannot be

dissociated under non-denaturing conditions (21,22). G-proteins not only process and sort signals but also define the sensitivity to the signal. As a result, G-protein signaling modulates many important physiological functions such as the pacemaker activity in the heart, development, and learning and memory (17,20,23). Heterotrimeric G-proteins act as molecular switches (Figure 1). In the inactive conformation, $G\alpha$ is bound to GDP at the seven transmembrane receptor and associates with the $G\beta\gamma$ dimer, which prevents the association of the α subunit with downstream effectors. Upon ligand binding to the seven transmembrane receptor, GDP is exchanged for GTP on the α subunit. This switches $G\alpha$ to the active conformation and results in the release of $G\alpha$ and $G\beta\gamma$ from the receptor. The conformational change in $G\alpha$ also leads to its dissociation from $G\beta\gamma$ and both molecules signal to downstream partners. The switch is turned off by the intrinsic GTP hydrolysis (GTPase) activity of $G\alpha$, which leads to its reassociation with $G\beta\gamma$ and the receptor. Two families of proteins, the regulators of G-protein signaling (RGS) and the activators of G-protein signaling (AGS), provide another layer of regulation of heterotrimeric G-protein signaling. RGS proteins greatly enhance the intrinsic GTPase activity of $G\alpha$ and act as GAPs (GTPase activating proteins) specific for $G\alpha$ (24). Thus, RGS proteins are crucial in inactivating G-protein signaling and are important regulators of the many processes controlled by G-proteins. In contrast, AGS proteins can activate G-proteins by two mechanisms, and their mode of action has not been fully clarified (25). While some AGS proteins such as AGS1 promote the exchange of GTP for GDP (26), others activate G-proteins independently of nucleotide exchange. AGS3, for instance, binds to GDP-bound $G\alpha$ and appears to prevent $G\alpha$ from reassociating with $G\beta\gamma$ (27,28).

Heterotrimeric G-proteins transduce many diverse signals into a wide assortment of cellular responses by coupling to their downstream effectors (23). Over 20 different $G\alpha$ proteins have been identified in the mammalian heterotrimeric G-protein superfamily, and the α subunits can be divided into four main families: G_s , G_q , $G_{i/o}$, and $G_{12/13}$ (20,29,30). Several $G\alpha$ proteins (G_s , G_i , and G_q) are expressed ubiquitously, while others such as G_o are restricted to specific tissues. Eight members of the $G_{i/o}$ family have been identified: $G_{\alpha_{i1}}$, $G_{\alpha_{i2}}$, $G_{\alpha_{i3}}$, G_{α_o} , G_{α_z} , $G_{\alpha_{\text{gust}}}$, $G_{\alpha_{t-c}}$, and $G_{\alpha_{t-r}}$. All of the members of the $G_{i/o}$ family except G_{α_z} are pertussis toxin sensitive. Several bacterial toxins including pertussis toxin disrupt the G-protein cycle shown in Figure 1 and are useful in dissecting the mechanism of G-proteins. These toxins ADP ribosylate the α subunit of specific G-proteins (31). For $G_{i/o}$, treatment with pertussis toxin leads to uncoupling of the receptor and the G-protein and inactivation of signaling. Under normal conditions, the binding of an extracellular ligand to its receptor results in activation of signaling pathways downstream of the G-proteins in order to elicit the desired cellular response (21). It is often difficult to functionally separate the downstream pathways activated by G_{α_i} and G_{α_o} . As a result, they are commonly named together as $G_{i/o}$.

In contrast to the well characterized pathways transduced by other G-proteins such as G_s , the mechanisms

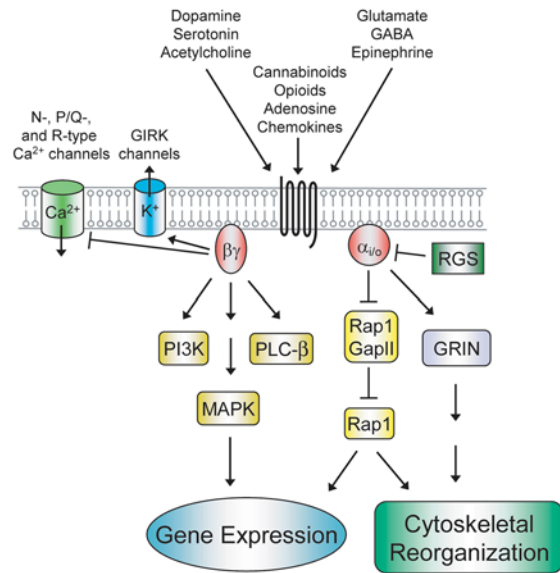


Figure 2. Effector pathways activated by G_{i/o} signaling. Signals from a wide array of hormones, neurotransmitters, and chemokines are transduced into intracellular responses by G_{i/o}-coupled receptors. Depicted are pathways that are stimulated by Gα and Gβγ and lead to changes in gene expression and cytoskeletal reorganization. See text for further details. GIRK, G-protein-coupled inward rectifying potassium channels.

by which G_{i/o} signals to its downstream effectors and induces cellular responses are not as well understood. The ubiquitously expressed Gα_i family members have been shown to inhibit several adenylyl cyclases (32). Gα_o is expressed abundantly in the brain and central nervous system, and it constitutes 0.5% of membrane protein in neurons (33,34,35). Gα_o is also expressed in lower levels in the pituitary, heart, and pancreas (33). In cellular and biochemical experiments, the Gβγ subunit of G_o has been shown to regulate numerous cellular processes including the inhibition of neuronal voltage-dependent Ca²⁺ channels (N-, P/Q-, and R-type) and the activation of inward rectifying K⁺ channels (36,37,38). G_o has also been shown to activate phospholipase C (PLC)-β (39), adenylyl cyclases 2 and 4 (40), and phosphoinositol-3-kinase (PI3K)-β and -γ (41,42); it has inhibitory effects on adenylyl cyclase 1 (40) and several voltage-gated Ca²⁺ channels (43,44).

In contrast, the function of the α subunit of G_o has remained elusive despite the fact that it is the most abundant G-protein in the brain. In Chinese Hamster Ovary (CHO) cells, Gα_o was shown to signal to p42/44 mitogen activated protein kinase (MAPK) through protein kinase C (PKC), although the functional significance of this was not known (45). It was also determined that Gα_o was highly expressed in the neuronal growth cone (46), the specialized structure at the tip of the growing neurite that is generated during neuronal differentiation (47,48). Strittmatter *et al.* (49) extended this finding by demonstrating that an activated mutant of Gα_o can induce neurite outgrowth in PC12 cells. The generation of Gα_o-deficient mice provided further insight into its function (50,51). Gα_o knockout mice were

smaller than their wild-type littermates and displayed a marked decrease in lifespan. They also exhibited hypersensitivity to pain, severe motor impairment, and occasional tremors and seizures. Still, the animals were hyperactive and displayed abnormal motor behavior as they ran in circles for extended periods of time. Studies of Gα_o knockout mice also indicated that Gα_o is important for muscarinic inhibition of L-type Ca²⁺ channels in the heart (50) and the regulation of Ca²⁺ and K⁺ channels in hippocampal neurons (52). Finally, Gα_o is required for cell survival in the accessory olfactory system, as the loss of Gα_o caused neuronal apoptosis and a decrease in G_o receptor containing neurons in the basal vomeronasal organ (53).

Only recently have studies begun to elucidate the mechanistic details of Gα_o signaling, and it has proven difficult to identify direct effectors of Gα_o. Several studies have demonstrated that Gα_o signaling activates a Src-STAT3 pathway that triggers cell transformation in NIH-3T3 fibroblasts (54,55). In addition, Gα₂ is important for inducing cell transformation in a Src- and STAT3-dependent manner in NIH-3T3 cells when these cells are transfected v-fms, an oncogenic form of colony stimulating factor (CSF)-1 receptor (56). In both of these cases, direct effectors of Gα remained unknown. To identify proteins that directly interact with Gα_o, our laboratory utilized a yeast two hybrid system (57). We found that Gα_o interacted with a G_z-GTPase activating protein (G_z-GAP), RGS protein 17 (RGS-17), a GTPase protein for the small G-protein Rap1 (Rap1GAP), and the G-protein regulator of neurite outgrowth (GRIN). G_z-GAP (also called RGSZ1) plays a role in attenuating mu opioid receptor inhibition of cAMP (58). RGS-17 has been shown to regulate G_{i/o}, G_z, and G_q signaling (59,60). Rap1GAP also interacts with Gα₂, and this interaction leads to a decrease in Rap1 activity (61). In thyroid cells, withdrawal of thyroid-stimulating hormone causes Rap1GAP proteasomal degradation that is glycogen synthase kinase (GSK)-3β dependent (62). The interaction between Gα_o and Rap1GAP was confirmed *in vitro* and in cultured cells, and Rap1GAP appears to preferentially bind to the inactivated form of Gα_o (63). In the case of Gα_o, the interaction with Rap1GAP leads to the activation of Rap1. Expression of Gα_o induces the degradation of Rap1GAP and treatment with proteasomal inhibitors blocks this effect, suggesting the degradation is ubiquitin-proteasome dependent. GRIN was concurrently identified in a mouse embryo expression library screen for proteins that bound to GTPγS-bound Gα_z (64). Two GRIN family members GRIN1 and GRIN2 bind specifically to Gα_o, and GRIN1 also binds to Gα_i and Gα_z. Similar to Gα_o, GRIN1 and 2 are also enriched in neuronal growth cones. The roles of Rap1GAP and GRIN in G_o signaling during neurite outgrowth are discussed below. The ligands that stimulate G_{i/o}-coupled receptors and the effectors that are activated are summarized in Figure 2.

4. INDUCTION OF NEURITE OUTGROWTH BY G_{i/o}

The identification of direct interactors with the α subunit of G_o raised several questions. What ligands might stimulate these interactions? What downstream signaling

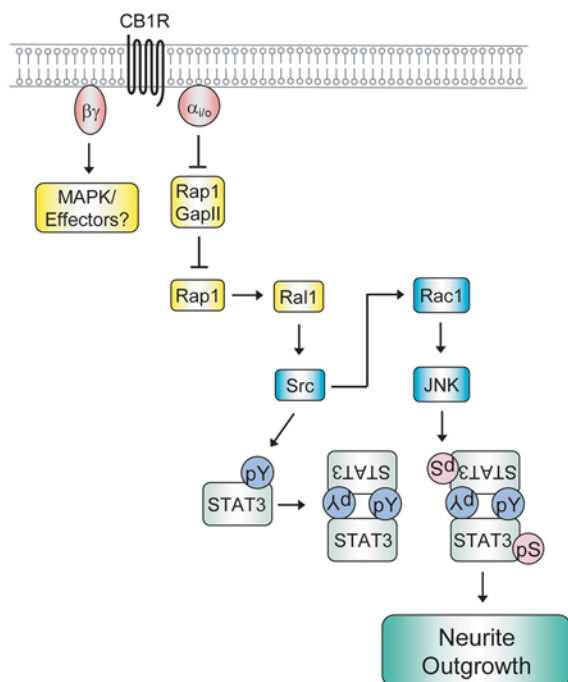


Figure 3. G_{i/o} signaling to the nucleus during the induction of neurite outgrowth. Signal flow emanating from stimulation of the G_{i/o}-coupled cannabinoid receptor 1 (CB1R) to the activation of the transcription factor STAT3 is depicted in the schematic. It is likely that Gβγ also signals to downstream effectors to change patterns of gene expression, possibly through p42/44 mitogen activated protein kinase (MAPK). See text for further details. pY, phospho-tyrosine; pS, phospho-serine.

pathways are subsequently activated? What is the cellular functional output of the signaling cascades? It was likely that G_{α_o} signaling stimulated neurite outgrowth. G_{α_o} and one of its interactors GRIN are both enriched in the growth cone of neurites (46,64). The collapse of the growth cone, which occurs when the extending neurite contacts variety of molecules (65,66), can be inhibited by pertussis toxin (67). Additionally, the expression of an activated mutant of G_{α_o} as well as the expression of GRIN induced neurite outgrowth (49,64). Rap1 had also been implicated in neurite outgrowth. Stable expression of the Src family member tyrosine kinase GTK stimulated neurite outgrowth in PC12 cells (68). This appears to be induced by signaling to focal adhesion kinase (FAK) through CrkII, and this in turn leads to phosphorylation of the adaptor protein Shb. Expression of Rap1GAP in this context inhibits GTK-induced neurite outgrowth, linking Rap1 to this pathway. Finally, NGF and EGF can induce neurite outgrowth in a Rap1 dependent manner in PC12 cells that overexpress the adaptor Shb (69).

Neurite outgrowth had been shown to be induced by several ligands that activate G_{i/o}-coupled receptors. Dopamine stimulation of the D2 dopamine receptor induces neurite outgrowth in cortical neurons (10) and serotonin activation of the serotonin-1B receptor enhances neurite outgrowth in thalamic neurons (11). In these cases, however,

the effectors and signaling pathways downstream of G_{α_o} had not been elucidated. Recently, our laboratory demonstrated that the cannabinoid receptor 1 (CB1R) stimulates G_{α_o} and activates downstream signaling converging on STAT3 that ultimately leads to neurite outgrowth in Neuro2A cells (14,63). In order to induce neurite outgrowth, signals from G_{α_o} are transduced to the nucleus to regulate gene expression and to the actin and microtubule cytoskeletal networks. Our current knowledge of G_{α_o} signaling to nucleus during neurite outgrowth is shown in Figure 3 and is discussed below. The following two sections will focus primarily on signal flow through the G_{i/o}-coupled CB1R, which is one of the best described systems for G_{i/o}-mediated neurite outgrowth.

4.1. Signaling to the nucleus

Heterotrimeric G-proteins transduce signals from the plasma membrane to the nucleus to change patterns of gene expression. During the past few years, several studies have begun to clarify the mechanisms by which activation of the G_{i/o}-coupled CB1R induces neurite outgrowth. Stimulation of CB1R with HU-210, a potent agonist for this receptor, induces neurite outgrowth (63). Either siRNA targeting Rap1 or dominant negative Rap1 is able to block this effect. This is consistent with the finding that activation of CB1R stimulates the interaction between G_{α_o} and Rap1GAPII, which results in the ubiquitin-proteasome mediated degradation of Rap1GAPII. Treatment with pertussis toxin or lactacystin also blocks Rap1GAPII degradation and inhibits CB1R-mediated neurite outgrowth. These results suggest that CB1R stimulates neurite outgrowth via the activation of Rap1 primarily through the attenuation of its inhibition by Rap1GAPII. However, the mechanistic details of Rap1GAPII degradation remain to be determined. Future studies will address whether G_{α_o} bridges an interaction between a currently unknown ubiquitin E3 ligase and Rap1GAPII. It is also possible that the binding to G_{α_o} causes a conformational change in Rap1GAPII which enables it to be recognized and degraded by the ubiquitin proteasome system.

Downstream of the activation of Rap1, G_{i/o}-coupled receptors activate Src and STAT3 (14). Both Src and STAT3 are phosphorylated in response to CB1R activation by HU-210, and this activation is pertussis toxin sensitive. In addition, dominant negative Src and STAT3 both strongly inhibit CB1R-mediated neurite outgrowth. Src and STAT3 appear to be activated by Rap1 as dominant negative Rap1 inhibits the phosphorylation of both proteins. Dominant negative Ral1 also inhibits Src and STAT3 activation and neurite outgrowth, suggesting a role for Ral in this pathway. Ral1 acts downstream of Rap1, as dominant negative Ral1 inhibits activated Rap1-mediated neurite outgrowth. Src is downstream of Ral1 since dominant negative Ral1 does not inhibit STAT3 activation mediated by v-Src. CB1R activation also stimulates the small GTPase Rac1 (discussed below in “4.2. Signaling to the cytoskeleton”) and c-Jun N-terminal kinase (JNK). Both of these proteins are activated downstream of Src, and JNK enhances STAT3 activation. Lastly, inhibition of JNK blocked the activation of STAT3 by Src and CB1R as well as CB1R-mediated neurite outgrowth.

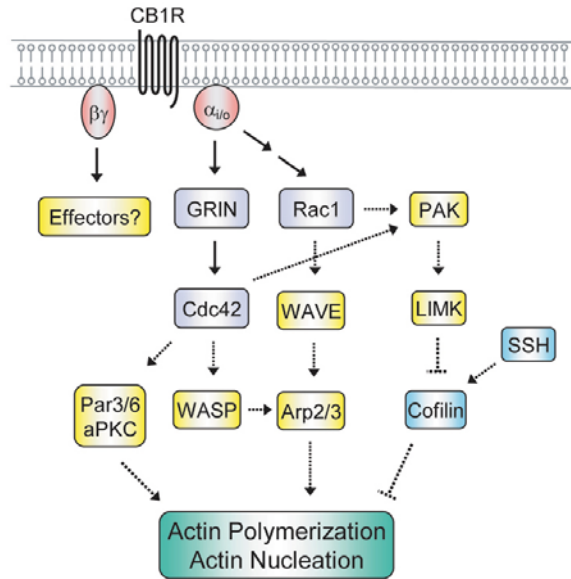


Figure 4. G_{i/o} signaling to the actin cytoskeleton during the induction of neurite outgrowth. Signaling from the G_{i/o}-coupled CB1R to its effectors GRIN, Cdc42 and Rac1 and their potential downstream targets is shown in the schematic. The intermediate molecules between CB1R and Rac1 have been omitted for clarity. It is likely that Gβγ also signals to yet to be identified downstream effectors to reorganize the actin cytoskeleton. Known interactions are depicted by solid arrows, putative interactions by dashed arrows. See text for further details. PAK, p21-activated kinase; SSH, Slingshot; WASP, Wiskott-Aldrich-syndrome protein.

In addition to CB1R signaling, activation of the G_{i/o}-coupled Serotonin (5-HT) 1 receptor induces neurite outgrowth in Neuro2A cells transfected with the 5-HT1A receptor and enhances cell survival in SK-N-SH cells endogenously expressing the receptor (70). Similar to CB1R, neurite outgrowth induced by the 5-HT1 receptor was pertussis toxin sensitive, required Rap1, and correlated with the phosphorylation of Src and STAT3. Dominant negative STAT3 strongly inhibited neurite outgrowth, whereas inhibition of p42/44 MAPK and PI3K led to a partial inhibition. Thus, it appears that several ligands that bind to distinct G_{i/o}-coupled receptors may induce neurite outgrowth by similar mechanisms.

While the signaling networks emanating from the CB1R and 5-HT1 receptors provide insight into G_{i/o} signaling during neurite outgrowth, it also opens several new avenues to explore. The genes that are transcriptional targets of STAT3 during neurite outgrowth remain to be identified. It is likely that CB1R stimulation leads to the activation of other transcription factors in addition to STAT3 and that different cell types may use different effectors to induce neurite outgrowth. STAT3 appears to play an important role in neurite outgrowth in response to G_{i/o} signaling in Neuro2A and SK-N-SH cells, but its contribution to neurite outgrowth in PC12 cells is less clear. IL-6 stimulation induces neurite outgrowth in PC12 cells in

a STAT3 dependent manner, and co-treatment of IL-6 and the neurotrophin NGF (nerve growth factor) leads to synergistic neurite outgrowth (12,71). However, a separate study found p42/44 MAPK activation downstream of the IL-6 receptor is essential for neurite outgrowth, while STAT3 appeared to have a negative regulatory role on neurite outgrowth (72). Recently, NGF stimulation has been shown to lead to the activation of STAT3 DNA binding and transcriptional activities (73). Inhibition of STAT3 expression led to a decrease in NGF induced gene transcription in PC12 cells and a decrease in neurite outgrowth induced by brain-derived neurotrophic factor (BDNF) in primary hippocampal neuron cultures. Similar to STAT3, the role of p42/44 MAPK in neurite outgrowth may vary with cell type. While stimulation of CB1R activates p42/44 MAPK in Neuro2A cells, its role in neurite outgrowth is unclear as p42/44 MAPK does not appear to be required for neurite outgrowth (14). However, p42/44 MAPK does appear to have an important role in response to 5-HT1 receptor signaling during neurite outgrowth (70). While p42/44 MAPK can also be activated by signaling through other G_{i/o}-coupled receptors such as the M₁ muscarinic acetylcholine receptor and the platelet-activating factor receptor (45), the functional relevance is not known. Thus, it appears likely that different signaling pathways may mediate similar effects in different cell types. Future studies will be necessary to elucidate the underlying molecular mechanisms and to delineate how multiple signals are integrated into transcriptional responses during neurite outgrowth. In addition to the α subunit, Gβγ also signals to downstream effectors upon receptor stimulation. The βγ subunit can activate several effector pathways, including MAPK, PI3K, and PLC (74). Currently, the contribution of βγ and its effector pathways that may be activated during neurite outgrowth remain to be determined.

4.2. Signaling to the cytoskeleton

During neurite outgrowth, the actin and microtubule cytoskeletal networks work in a coordinated fashion to generate and stabilize the growing neurites (1,8). The actin cytoskeleton reorganizes to allow formation of the growth cone and the microtubules re-align into bundles to stabilize the growing neurite. The Rho family of small G-proteins plays a key role in the cytoskeletal reorganization that occurs during the initiation, guidance, and elongation of the neurite (1). Twenty two genes encoding mammalian Rho GTPase family members have been identified (1,75). The three best described Rho GTPases RhoA, Rac1, and Cdc42 regulate the cytoskeleton and a myriad of cellular functions including cell polarity, gene transcription, cell cycle, enzyme activity, apoptosis and vesicle transport (75,76). On the biochemical level, Rho regulates the actin-myosin contractile apparatus and stress fiber formation, Rac regulates the formation of web-like lamellopodia protrusions and membrane ruffling, and Cdc42 regulates the protrusions of finger-like filopodia. In order to generate the growth cone and initiate neurite formation, signals are transduced at the membrane through the Rho family of GTPases to the actin cytoskeleton. In general, neurite outgrowth is stimulated by Rac and Cdc42 activation, while Rho activation appears to promote neurite

retraction (1). The mechanisms by which G_{i/o} may signal to the actin cytoskeleton are discussed below and summarized in Figure 4.

The molecular mechanism of G_{i/o} stimulated cytoskeletal signaling is just beginning to be elucidated. The G_{α_o} interactor GRIN is enriched in the growth cone, and co-expression of GRIN with an activated mutant of G_{α_o} enhances neurite outgrowth in Neuro2A cells (64). Co-expression of these two proteins also leads to activation of Cdc42, and neurite outgrowth under these conditions is blocked by dominant negative Cdc42 and dominant negative Rac1 (77). It is possible Cdc42 may mediate its effects by activating the Par6-Par3-atypical PKC (aPKC) complex (78). In hippocampal neurons that are plated in culture, Cdc42 and the Par6-Par3-aPKC complex are enriched in the developing axon. Alterations in this signaling axis lead to defects in neuronal differentiation, causing cells to produce either no or multiple axons (79). Other signaling molecules may also be downstream of Cdc42, and the signals that lead to Cdc42 activation and the downstream molecules that lead to cytoskeletal reorganization remain to be elucidated.

Our laboratory has linked Rac1 to neurite outgrowth induced by CB1R activation (14). Stimulation of CB1R leads to the activation of Rac1 that is Src dependent and expression of dominant negative Rac1 inhibits neurite outgrowth in Neuro2A cells. Rac1 is important for JNK activation, which in turn enhances Stat3 transcriptional activation and neurite outgrowth. In addition to these effects, Rac1 is likely to play an important role in cytoskeletal signaling during CB1R-mediated neurite outgrowth. Both Rac1 and Cdc42 can activate the p21-activated kinase (PAK) family of serine/threonine kinases (1,75). PAK can signal to the actin cytoskeleton by phosphorylating and activating the LIM kinases (LIMKs), which in turn phosphorylate and deactivate cofilin and its closely related protein, actin depolymerizing factor (ADF). Cofilin and ADF act by stimulating the depolymerization and severing of actin filaments (80,81) and their phosphorylation at Serine-3 by LIMKs results in actin polymerization. The Slingshot (SSH) phosphatases alleviate the inhibition of cofilin and ADF through dephosphorylation (82). Cofilin and ADF as well as their regulators LIMKs and SSHs are enriched in neuronal growth cones (1,83,84,85), and thus have been implicated in regulating actin cytoskeletal reorganization during neurite outgrowth. This regulation of actin dynamics is tightly regulated as the inhibition of cofilin/ADF, LIMK, or SSH inhibits neurite outgrowth (86). Rac1 and Cdc42 also can mediate their effects through the Wiskott-Aldrich-syndrome family of proteins (WASP). Rac1 can indirectly trigger the WASP family member WAVE to activate Arp2/3, which stimulates actin nucleation and the formation of new actin filaments (87,88). Similarly, Cdc42 can directly activate WASP to interact with and stimulate Arp2/3 (89). Future studies will examine whether G_{i/o}-coupled receptor stimulation activates Cdc42 and Rac1 to regulate PAK signaling to cofilin/ADF and WASP signaling to Arp2/3.

Might G_{i/o} also regulate RhoA activity during neurite outgrowth? The G_{i/o} effector Rap1 has been shown to activate the RhoGAP RA-RhoGAP (90). RA-RhoGAP is localized in the neuronal growth cone and Rap1 enhances the GAP activity of RA-RhoGAP toward RhoA. Rap1 activation of RA-RhoGAP also promotes neurite outgrowth in NG108 cells. Overexpression of another RhoGAP, p190 RhoGAP can induce neurite outgrowth in Neuro2A cells (91). In the developing and mature nervous system, p190 RhoGAP is phosphorylated by Src. This modification is likely to activate p190 RhoGAP, as phosphorylation of p190 RhoGAP is important for its binding to RhoA (92). It remains to be determined whether signals from G_{i/o} modulate RhoA during neurite outgrowth and whether Rap1 or other signaling components regulate this activity. The precise mechanisms of actin cytoskeletal regulation by G_{i/o} as well as deciphering G_{i/o} signaling to the microtubule network will be addressed by future studies.

5. PHYSIOLOGICAL AND PATHOLOGICAL IMPLICATIONS

5.1. Cannabinoid signaling during development

Cannabinoid signaling has important functions during central nervous system (CNS) development and in the postnatal brain. Endogenous cannabinoids (endocannabinoids) regulate synapse activity postnatally and regulate progenitor cell proliferation and the migration, differentiation, and survival of neurons during development (93,94). Recent studies of endocannabinoid ligands, their receptors, and downstream signaling have begun to clarify the mechanisms of cannabinoid signaling *in vivo* and provide insight into how exogenous cannabinoids affect these functions and may be used as therapeutics for several chronic diseases. The mechanisms by which endocannabinoids signal through the CB1R to shape CNS development are discussed below.

The endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are endogenous ligands for the G_{i/o}-coupled CB1R (reviewed in (94)). In the developing mouse CNS, CB1R expression appears to begin around embryonic day 11 (E11) in early progenitors and increases throughout the whole brain until birth. At E13.5, CB1R is expressed on pyramidal cells in the hippocampus and developing cerebral cortex primarily on distal parts of the axons (95). CB1R is also highly expressed in migrating GABAergic interneurons in the hippocampus and cortex. By E18, migrating GABAergic interneurons express CB1R on axons and at axonal growth cones (95). In this context, signaling through CB1R helps to shape axon guidance in the developing cortex. A recent study has determined that CB1R-deficient mice display defects in post-synaptic targeting in cortical interneurons (95). Endocannabinoids may regulate axon guidance by causing the internalization and retrograde transport of CB1R, which is concomitant with the activation of p42/44 MAPK. RhoA is also activated and its activation induces growth cone collapse. Inhibition of the Rho effector ROCK during this process can convert repulsion and collapse of the growth cone into chemoattraction. Thus, there may be fine-tuning of

endocannabinoid signaling to precisely regulate axonal target selection. In addition to regulating synaptic targeting, endocannabinoid signaling plays an important role in neuronal migration and morphogenesis. During morphogenesis, GABAergic cortical interneurons migrate long distances before reaching specific cortical layers, and it has been shown that BDNF helps determine the specification of cortical interneurons (96,97). The endocannabinoid AEA cooperates with BDNF to induce interneuron migration by transactivating the TrkB receptor through Src kinase activation (98). In fact, CB1R stimulation leads to TrkB receptor activation in a Src-dependent manner in the absence of BDNF, and CB1R and TrkB form complexes during AEA stimulation. In contrast, AEA inhibits BDNF-induced differentiation. Thus, signaling through CB1R helps to govern BDNF-induced migration and differentiation during corticogenesis.

Signaling through CB1R also regulates neural progenitor cell proliferation and differentiation. Proliferation of progenitor cells is promoted by CB1R activation, and this effect is attenuated in CB1R knockout mice (99,100). Endocannabinoids also regulate differentiation of progenitors into glia as loss of CB1R negatively impacts gliogenesis *in vivo* (100). In contrast, CB1R signaling may inhibit progenitor differentiation into neurons (101). Treatment with AEA inhibits cortical neuron progenitor differentiation and NGF-induced differentiation in PC12 cells. CB1R stimulation may inhibit neural progenitor cell differentiation by dampening signaling through the Rap1-B-Raf-p42/44 MAPK pathway as AEA blocked sustained p44/22 MAPK activation. Thus, in contrast to morphogenesis, CB1R stimulation can block Trk receptor activation during differentiation. The importance of proper regulation of endocannabinoid signaling during development is underscored by the prenatal exposure to Δ^9 -tetrahydrocannabinol (THC), the major psychoactive component of marijuana (*Cannabis sativa*). Several studies have indicated that maternal consumption of marijuana influences behavior in offspring, causing hyperactivity, inattention and cognitive defects by adolescence (102,103,104). THC also increases the synthesis and release of endocannabinoids in a pertussis sensitive manner, thus linking G_{i/o}-coupled receptors to its effects (105). In the developing hippocampus, blocking CB1R stimulation can lead to epileptic discharges, while over-activation can reduce network activity (106). Disturbing this balance has the potential for profound consequences during development. Indeed, sustained prenatal exposure to THC *in vivo* increases the density of CB1R-positive GABAergic interneurons in the hippocampus (98). These studies demonstrate that THC can alter the patterning of CB1R expressing cells and provide the framework to delineate the mechanism by which prenatal exposure to THC causes long-lasting effects.

5.2. Neurodegeneration

Beyond its regulation of neurite outgrowth and neurogenesis during development, signaling through G_{i/o} and its coupled receptors may possess neuroprotective and anti-inflammatory properties. Cannabinoid receptor

stimulation can protect hippocampal neurons from excitotoxicity (107,108,109). Furthermore, CB1R stimulation *in vivo* decreases hippocampal neuron loss after cerebral ischemia and acute brain trauma (110). Cannabinoids may also help protect neurons from alteration in glucose levels (111). Hyperglycemia plays a critical role in the development and progression of diabetic neuropathy, which is a complication that arises from diabetes mellitus and causes cognitive deficits. In a mouse model of diabetes, cannabinoid treatment ameliorates the cognitive impairment in these mice without altering the underlying hyperglycemia. However, these effects appear to be independent of CB1R, as the CB1R antagonist SR141716A did not affect the cognitive improvements (112). Cannabinoids are also important in modulating inflammation (113) and are being pursued as potential therapies for obesity (114,115). In addition, signaling through several G_{i/o}-coupled receptors has been shown to modulate a spectrum of neurodegenerative disease and may represent an attractive target for therapeutic intervention (116,117). The roles of G_{i/o}-coupled CB1R and adenosine receptors in neurological disorders are discussed below and summarized in Table 1.

Even though the endocannabinoid system is just beginning to be unraveled, it is being pursued as a therapy for many CNS disorders. In addition to cannabinoid signaling, several drugs targeting cannabinoid metabolism have shown efficacy at alleviating the symptoms of several animal models of neurodegenerative diseases (reviewed in (117,118)). In Parkinson's disease, CB1R agonists can prevent dopaminergic neuron loss (119). However, agonist-mediated stimulation of non-cannabinoid receptors can also promote neuronal apoptosis, demonstrating the need for strong receptor selectivity in potential therapeutic applications (120). Similarly, CB1R stimulation is detrimental to motor neuron survival in Amyotrophic Lateral Sclerosis while activation of the related family member type 2 cannabinoid receptor (CB2R) is neuroprotective (121,122). In Alzheimer's disease, stimulation of CB1R is protective against neurodegeneration (123). A similar prevention of degeneration is also observed by inhibiting the catabolism of AEA, thus increasing the pool of available endocannabinoids (124). Activation of CB1R is neuroprotective in animal models of multiple sclerosis and animals with CB2R genetically ablated in T cells display a severe disease phenotype (125). Neuroprotective effects in multiple sclerosis disease models are also observed by inhibiting the catabolism of AEA (126,127). Lastly, CB1R agonists as well as inhibitors of cannabinoid catabolism exhibit neuroprotective properties in models of Huntington's disease (118,128,129). As our understanding of the endocannabinoid system and the molecular details of the downstream signaling from the cannabinoid receptors continues to expand, new agents that selectively target the signaling through specific receptors as well as the synthesis and degradation of endocannabinoids will be developed for the treatment of neurodegenerative diseases.

Similar to CB1R, the G_{i/o}-coupled A1 adenosine receptor regulates neurite outgrowth and also represents a potential target for the treatment of CNS disorders.

Table 1. Potential therapeutics targeting G_{i/o}-coupled receptor signaling for the treatment of neurological disorders

Compound(s)	G _{i/o} -coupled Receptor	Indication	Mechanism of Action	Clinical Status	Reference
2-AG ¹ ; WIN 55212-2	CB1R	Cerebral ischemia/acute brain trauma	Receptor agonists	Animal Model	110,111
HU-210	ND ⁶	Diabetic neuropathy	CB1R independent	Animal Model	112
Δ ⁹ -THC ²	CB1R	Parkinson's Disease	Receptor agonist	Animal Model	119
Cannabidiol	ND	Parkinson's Disease	CB1R independent	Animal Model	119
HU-210; WIN 55212-2; JWH-133	CB1R	Alzheimer's Disease	Receptor agonists	Animal Model	123
VDM-11	CB1R	Alzheimer's Disease	endocannabinoid cellular reuptake inhibitor	Animal Model	124
Δ ⁹ -THC; WIN 55212-2	CB2R	Amyotrophic Lateral Sclerosis	Receptor agonists	Animal Model	121,122
Δ ⁹ -THC	CB1R	Multiple Sclerosis	Receptor agonist	Animal Model	125
OMDM1; OMDM2	CB1R	Multiple Sclerosis	endocannabinoid cellular reuptake inhibitors	Animal Model	126,127
CP55,940	CB1R	Huntington's Disease	Receptor agonist	Animal Model	128
AM404	CB1R	Huntington's Disease	endocannabinoid cellular reuptake inhibitor	Animal Model	128,129
FR194921	A1AR	Anxiety Dementia	Receptor antagonist	Animal Model	137
T-62	A1AR	Migraine headaches, chronic pain	Allosteric enhancer	Phase II Clinical Trials in U.S.A.	140
ADAC ³ ; NNC 21-0041,9; NNC 90-1515,4	A1AR	Cerebral ischemia	Receptor Agonists	Animal Model	142-144
IB-MECA ⁴	A3AR	Cerebral ischemia	Receptor agonist	Animal Model	145
CHA ⁵ ; adenosine	A1AR	Seizure	Receptor agonists	Animal Model	147,150

¹2-AG, 2-arachidonoylglycerol; ²Δ⁹-THC, delta9-tetrahydrocannabinol; ³ADAC, adenosine amine congener; ⁴IB-MECA, N6-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide; ⁵CHA, N6-cyclohexyladenosine; ⁶ND, not determined

Adenosine receptors are one of the main targets in the brain for caffeine, and there are several agonists and antagonists currently being evaluated for clinical use (116). Four subtypes of adenosine receptors have been identified, A1, A2A, A2B, and A3. In addition to A1, A3 also couples to G_{i/o}. Stimulation of A1AR and A3AR have long been known to inhibit adenylyl cyclase and subsequent cAMP production (130), and more recently they have been shown to activate other signaling molecules including phospholipases C and D, RhoA, p42/44 MAPK, and PI3K (131,132,133,134). On the cellular level, both A1AR and A3AR inhibit adenylyl cyclase and activate phospholipase C and p42/44 MAPK pathways (130,133). For A1AR, this results in the activation of pertussis toxin-sensitive K⁺ channels and inhibition of Q-, P- and N-type Ca²⁺ channels (135), which as discussed above are known effects of G_{i/o} signaling. Signaling through A3AR has protective functions against ischemia in cardiac tissue (136) and both A1- and A3AR regulate cell growth, survival, and differentiation (reviewed in (116,133)).

The discovery that caffeine exerts its stimulatory effects upon the CNS via antagonism of adenosine receptors has spurred the pursuit of modulators of these receptors to treat neurological disorders (116). Many agonists and antagonists of adenosine receptors have been generated as well as allosteric enhancers of receptor agonists. The latter molecules bind to the receptor in an area that is distinct from the agonist binding site and enhance the response of the receptor in the presence of the agonist. Antagonism of A1AR has shown promise in the treatment of anxiety and dementia as treatment with an A1AR antagonist reduced memory deficits in an animal model of dementia and had anxiolytic properties (137). Signaling through A1AR has also been shown to modulate pain by inhibiting cAMP production. A1AR-deficient mice

display hypersensitivity to pain and increased anxiety, suggesting that A1AR may be important in regulating chronic pain (138,139). Stimulation of A1AR may be useful in the treatment of migraine headaches (140), and the A1AR allosteric enhancer T-62 is in Phase II clinical trials for the treatment of chronic pain. The selective modulation of A1AR may also have neuroprotective effects as adenosine can counteract the toxic effects of glutamate-mediated excitotoxicity (141). Indeed, several A1AR agonists display neuroprotective properties in animal models of cerebral ischemia (142,143,144). In addition, stimulation of A3AR is also neuroprotective in a gerbil model of ischemia (145). Finally, as adenosine can inhibit excitatory neural activity, it is thought that signaling through adenosine receptors may inhibit seizures in the healthy brain (146). A1AR agonists can inhibit seizures in rats, and this effect is markedly decreased by an A1AR antagonist (147). Thus, the improper regulation of adenosine signaling can potentially aggravate existing seizures, and adenosine receptor agonists represent an attractive drug for therapeutic intervention (146,148). While efforts at the potential treatment of seizures have been hampered by side effects on the cardiovascular system including heart rate and blood pressure, several studies using local delivery methods have shown promise for the future (149,150).

6. PERSPECTIVE

Since the discovery over a decade ago that Gα_o induces neurite outgrowth, there has been rapid progress in elucidating the G_{i/o}-coupled receptors and the ligands that stimulate signaling through the system. The identification of Rap1GAPII as a direct interactor of Gα_o led to the delineation of a central signaling network from CB1R to STAT3 that controls cannabinoid-induced neurite

outgrowth. Several $G_{i/o}$ -coupled receptors have now been identified that upon stimulation induce neurite outgrowth with STAT3 emerging as a key signaling focal point. While it is clear that Rac1 and Cdc42 are central players in cytoskeletal reorganization during neurite outgrowth, future studies will elucidate the mechanistic details leading to actin and microtubule reorganization. These findings provide the foundation for tackling the next set of challenges. These include defining the target genes activated in response to $G_{i/o}$ signaling, delineating their transcriptional regulation, and determining how signals that stimulate other receptor-based signaling systems are integrated into this network to induce neurite outgrowth. Indeed, steps have already been taken for the latter, as signals through both the $G_{i/o}$ -coupled CB1R and the TrkB receptor are integrated for proper corticogenesis during development (98). Clarifying these signaling networks will also provide a framework for delineating $G_{i/o}$ signaling during axonal growth cone pathfinding and collapse as well as axonal regeneration. While several studies have implicated $G_{i/o}$ -coupled receptors in these processes (46,64,67,151,152,153), the molecular mechanisms remain mostly undiscovered. Deciphering the signaling networks that regulate $G_{i/o}$ -mediated neurite outgrowth from the level of receptor activation to gene expression will yield great insight into the regulation of neurite outgrowth, how this system is modulated physiologically during development and in the adult, and how it can be exploited for therapeutic intervention.

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